Parasite Immunology, 2006, **28**, 221–228 DOI: 10.1111/j.1365-3024.2006.00830.x

Bovine splenic NK cells synthesize IFN-γ in response to IL-12-containing supernatants from *Babesia bovis*-exposed monocyte cultures

WILL L. GOFF,[1] ANNE K. STORSET,[2] WENDELL C. JOHNSON[1] & WENDY C. BROWN[3]

[1]Animal Disease Research Unit, USDA-ARS, Pullman, Washington, USA, [2]Food Safety and Infection Biology, Norwegian School of Veterinary Science, Oslo, Norway, [3]Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA

SUMMARY

The spleen is a critical effector organ functioning, in haemoparasitic diseases like babesiosis, to destroy the pathogen and clear the host of infected erythrocytes. It has an important role in both innate responses and adaptive immune responses. Young calves demonstrate a strong spleen-dependent innate response to an initial infection with Babesia bovis *involving the type-1 regulating cytokines IL-12 and IFN-γ. However, the specific splenic cell types that produce IFN-γ in response to infection and the cellular factors that regulate the induction have not been fully determined. Splenic NKp46$^+$ NK cells were identified and purified. They consisted of CD3$^-$, CD2$^{+/-}$, and CD8$^{+/-}$ populations. NK cells responded to exogenous IL-12 and IL-18 with the production of IFN-γ. Functionally, IL-18 served as a potent co-stimulant with IL-12 for IFN-γ production. Finally, innate IFN-γ production was induced in splenic NK cells in the presence of supernatants from* B. bovis *merozoite-exposed monocytes in an IL-12 pathway-dependent manner.*

Keywords Babesia*, bovine, IFN-γ, innate immunity, NK cells, regulatory cytokines*

INTRODUCTION

Interferon gamma (IFN-γ) is a critical cytokine in the innate immune response of cattle to intracellular pathogens, including *Babesia bovis*. Adult cattle are more susceptible to severe disease caused by *B. bovis* than young calves that control the acute parasitaemia via a spleen-dependent innate immune response characterized by the early induction of IL-12 and IFN-γ (1). IFN-γ activates monocytes for increased microbicidal activity (2,3) and regulates the synthesis of opsonic IgG_2 from bovine B-cells (4). Activated monocytes/macrophages function as regulatory cells (2,5) as well as effector cells. As effector cells, they produce babesiacidal nitric oxide and phagocytose live and dead merozoites and infected erythrocytes (6–8). Both monocytes/macrophages and dendritic cells (DC) produce a myriad of regulatory cytokines, including IL-12, IL-15 and IL-18 (9–11), each synergistically contributing to the induction of IFN-γ production (12,13) from a variety of cells including Type 1 CD4$^+$ and CD8$^+$ αβ T-cells, γδ T-cells, and natural killer (NK) cells.

NK cells are innate effector cells first recognized by their ability to lyse tumour cells without previous activation (14). NK cells discriminate between normal cells and cells that no longer express MHC class I due to viral infection or tumour transformation i.e. cytolytic killing of non MHC class I bearing target cells. This discrimination results from the interaction of killer inhibitory receptors with class I ligands (15). In addition to their cytolytic function, there is evidence that a subpopulation of human NK cells, enriched in secondary lymphoid tissue, serve a regulatory function and participate in innate type-1 responses by producing IFN-γ (12). They have been shown to respond to cytokine-producing DC *in vitro* (16,17), *ex vivo* from lymph nodes, and at sites of inflammation (18).

NKp46 is an activating natural cytotoxic receptor expressed on most or all human NK cells (19). NKp46 has recently been demonstrated on a bovine lymphocyte population with

Correspondence: W. L. Goff, Animal Disease Research Unit, USDA-ARS, 3003 ADBF, Washington State University, Pullman, WA 99164-6630 USA (e-mail: wgoff@vetmed.wsu.edu).
Received: 7 October 2005
Accepted for publication: 20 December 2005

NK characteristics and specific reagents were described for use in defining and purifying this important cell population (20). In addition, we recently demonstrated a $CD3^-$, $CD2^+$, $CD8^+$ NK-like cell proliferating in the spleens of calves during the response to an initial infection with *B. bovis* (21). Therefore, it was of interest to determine if a splenic $NKp46^+$ cell could respond to pro-inflammatory cytokines with the production of IFN-γ. Evidence is presented that suggests NK cells may play a significant role in the type-1 innate immune response of cattle to *B. bovis* infection, and that this is an important source of local IFN-γ production.

MATERIALS AND METHODS

Source of splenic leucocytes

Six Holstein-Friesian calves were obtained at 8 weeks of age and maintained according to the American Association for Laboratory Animal Care procedures with an acceptable bovine ration, water and mineral block provided *ad libitum*. The spleen of each animal was surgically marsupialized to facilitate aspiration of spleen cells (22). The procedure has proven to be a means of acquiring sequential samples from a single animal without demonstrable consequences to splenic phenotypic ratios or basal levels of cellular activity (5). Splenic aspirates were aseptically collected in 60 mL syringes containing 15 mL acid–citrate–dextrose (ACD) pH 7·3 under local anaesthesia and processed into a single-cell suspension using a tissue homogenizer. Splenic cell suspensions were layered onto Hypaque-Ficoll (1·086 g/L) (Accu-Paque, Accurate Chemicals, Westbury, NY) and centrifuged for 30 min at 1500 ***g*** at 4°C. Splenocytes from similar gradients were collected, pooled, and washed in 50 mL Dulbecco's modified eagle's medium (DMEM), pH 7·2, for 7 min at 1500 ***g*** at 4°C. The cells were suspended with DMEM and washed twice at 400 ***g*** for 7 min and 4°C to remove platelets. The final pellet was suspended to 1×10^7 cells/mL in Iscove's medium (Gibco BRL, Gaithersburg, MD) containing 25 mM Hepes, 2 mM glutamine, 10 μg/mL gentamycin, 50 μM mercaptoethanol, and 15% essentially endotoxin-free foetal bovine serum (FBS) (< 0·06 EU/mL as assayed by Limulus amoebocyte lysate gelation) (Hyclone, Logan, UT).

Monoclonal antibodies used in flow cytometric analysis

Several leucocyte differentiation determinate-specific monoclonal antibodies (MoAb) were used to define splenic cell phenotypes. They included: MUC2A (CD2, IgG_{2a}), BAQ111A (CD8 alpha, IgM), CACT108A (CD25, IgG_{2a}), GB21A (TcR1, IgG_{2b}), B7A1 (WC1, IgM) (VMRD, Pullman, WA); IL-A11 (CD4, IgG_{2a}) (gift from Dr Chris Howard, Institute for Animal Health, Compton, UK); and AKS1 (NKp46, IgG_1) (20) (provided by author A.S. from this report).

Purification of $NKp46^+$ cells

For some experiments, $NKp46^+$ cells were purified from splenocytes via MagCellect™ with Streptavidin Ferrofluid™ (R&D Systems, Minneapolis, MN) per the manufacturer's protocol. Briefly, 1×10^6 splenocytes were washed into phosphate buffered saline supplemented with 0·5% BSA, 2 mM EDTA (PBS-BE), suspended to 1 mL and incubated with 6 μg AKS1 for 15 min at 4°C. The cells were washed once with PBS-BE at 400 ***g*** at 4°C. Cells were suspended to 1 mL with PBS-BE and incubated as before with 10 μL goat anti-mouse IgG + IgM-biotin (Caltag Laboratories, Burlingame, CA) and then washed as before. Cells were suspended in 900 μL PBS-BE, 100 μL Streptavidin Ferrofluid was added, and the cells incubated as before. Labelled cells were diluted to 2 mL with PBS-BE and captured three times for 6 min each using a MagCellect magnet. Isolated cells were washed into Iscove's/FBS prior to use.

In vitro enrichment of $NKp46^+$ cells

For experiments using highly enriched $NKp46^+$ cells, splenocytes at 1×10^6/mL in Iscove's/FBS containing either 20 ng/mL rHuIL-15 (R&D Systems) or 2·5 U/mL rBoIL-2 (gift of Dr Nancy Magnuson, Washington State University) were incubated for 14 days at 37°C and 5% CO_2. rBoIL-2 activity was determined by a cell proliferation assay using bovine IL-2-dependent lymphocyte cultures as assay cells. One unit of activity was defined as the amount of IL-2 producing 50% of maximal thymidine incorporation induced by recombinant human IL-2. Prior to use, cells were collected on Hypaque-Ficoll as described above, washed into Iscove's/FBS and suspended to a concentration of $1–10 \times 10^6$ cells/mL. The cell purity of isolated or enriched cells was determined by one-colour flow cytometry with AKS1 MoAb and anti-mouse IgG1 – Tricolor (Caltag Laboratories).

Induction of interferon-γ

To evaluate the ability of splenocytes and purified or enriched $NKp46^+$ cells to produce IFN-γ, cells were incubated overnight in combinations of 20 ng/mL rHuIL-12 with or without 200 pg/mL (suboptimal) or 1000 pg/mL rHuIL-18 at 37°C and 5% CO_2. To evaluate the ability of *B. bovis* to stimulate IFN-γ production in $NKp46^+$cells via IL-12, bovine adherent monocytes and *B. bovis* merozoites were isolated as previously described (5). rHuIL-12 was replaced with culture supernatants from 5×10^5 monocytes/mL incubated overnight with or without 5×10^6 *B. bovis* merozoites/mL. Monocyte production of IL-12 p40 was evaluated by a previously described dot blot

technique (23) using a normalized human IL-12 standard curve. In an attempt to neutralize IL-12 activity from monocyte supernatants, CC301 MoAb (provided by Dr Jayne Hope, Compton, UK) ranging from 3 to 12 μg was added to 100 μL of supernatants, incubated for 30 min at room temperature and then added to NK cell cultures overnight as above. Specific involvement of the p38 MAPK pathway was determined by incubating cells with the inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) (Sigma, St. Louis, MO) (24) at a concentration of 10 μM for 30 min at room temperature prior to exposure to IL-12, IL-18 or monocyte supernatant.

Evaluation of interferon-γ production

Identification of cells producing IFN-γ was accomplished by three-colour flow cytometry. Cells stimulated overnight were incubated for 4 h with Golgi Plug™ (Becton Dickinson, San Jose, CA) to inhibit transport of synthesized IFN-γ from the Golgi complex. Cells were stained for surface-exposed determinates using an appropriate combination of monoclonal antibodies listed above. After washing, labelled cells were stained with an appropriate combination of secondary fluorescent conjugates (anti-IgM-PE, anti-IgG_{2a}-PE (Southern Biotechnology Associates, Birmingham, AL), anti-IgG_1-Tricolor and anti-IgG_{2b}-Tricolor (Caltag Laboratories)). Cells were washed and then fixed with Cytofix/CytoPerm™ (Becton Dickinson) for internal staining of accumulated IFN-γ. Permeablized cells were incubated with anti-bovine IFN-γ-FITC (MCA1783-F) (Serotec, Raleigh, NC), washed with Perm/Wash™ solution (Becton Dickinson) and evaluated with FACScan flow cytometry (Becton Dickinson). Soluble IFN-γ produced by enriched $NKp46^+$ cells stimulated overnight with monocyte supernatants plus suboptimal rHuIL-18 were evaluated by BoviGam™ (Biocor Animal Health, Omaha, NE) enzyme immunoassay per the manufacturer's protocol.

RESULTS

Bovine spleen cells include subpopulations that respond to IL-12 and IL-18 with the production of IFN-γ

In preliminary experiments to determine whether exogenous pro-inflammatory cytokines can induce IFN-γ production from bovine spleen cells, total spleen cells were cultured overnight in the absence or presence of IL-12, IL-18 or the combination of the two. IL-12 alone and IL-18 alone stimulated a small proportion of spleen cells to produce a modest amount of IFN-γ (Figure 1b,c). However, IFN-γ production was increased dramatically in response to the combination, suggesting that IL-18 acted as a synergistic co-stimulant with IL-12 (Figure 1d), as previously demonstrated for bovine PBMC (25). Among the phenotypes producing IFN-γ were subpopulations of NK cells, $CD4^+$ cells, $CD8^+$ cells, $CD2^+$ cells, and γδ T-cells (Figure 2a–f). In γδT-cells, the IFN-γ production was restricted to a $WC1^-$ subpopulation that predominates in ruminant spleen.

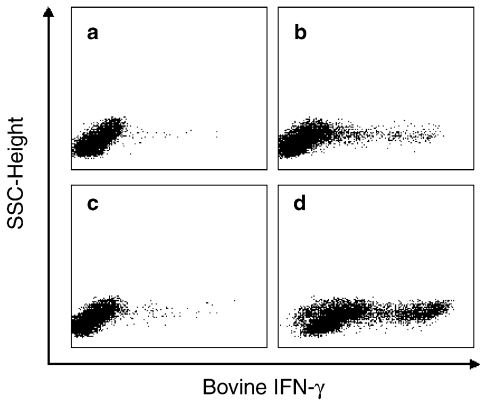

Figure 1 IFN-γ production from cytokine-stimulated bovine spleen cells. Fresh, bovine spleen cells were internally labelled with FITC-anti-bovine IFN-γ for flow cytometric analysis after overnight stimulation with 20 ng/mL rHuIL-12 and/or 1000 pg/mL rHuIL-18. (a) control, unstimulated spleen cells; (b) spleen cells stimulated with IL-12 alone; (c) spleen cells stimulated with IL-18 alone; (d) spleen cells stimulated with IL-12 plus IL-18. Resulting plots are representative of all calves.

Effect of IL-2 and IL-15 on NK proliferation and receptor expression

Bovine spleen cells were cultured for up to 14 days in the presence of 2·5 U/mL of bovine rIL-2 or 20 ng/mL human rIL-15 to determine if splenic NK cells would proliferate and increase NKp46 expression. The effect of bovine rIL-2 or human rIL-15 on bovine spleen cells was similar, providing for 90% enrichment of NK cells. NK cells increased in numbers and expression of NKp46 on a per cell basis (Figure 3a). Typically, total numbers of $NKp46^+$ cells obtained at the end of 2 weeks increased > 10-fold with γδ T-cells making up most of the contaminating non-NK population (data not shown). As expected, both IL-2 and IL-15 resulted in the increased expression of CD25, the α subunit of the IL-2 receptor (Figure 3b).

Like fresh NK cells from blood (20), the majority of fresh splenic NK cells expressed CD2 (Figure 4a, panel 1). In contrast to blood-derived NK cells, where IL-2 activation resulted in proportionately fewer $CD2^+$ NK cells, IL-2 or IL-15 activated splenic NK cells were predominantly $CD2^+$, $CD8^-$ (Figure 4a, panels 2–4, and 4b).

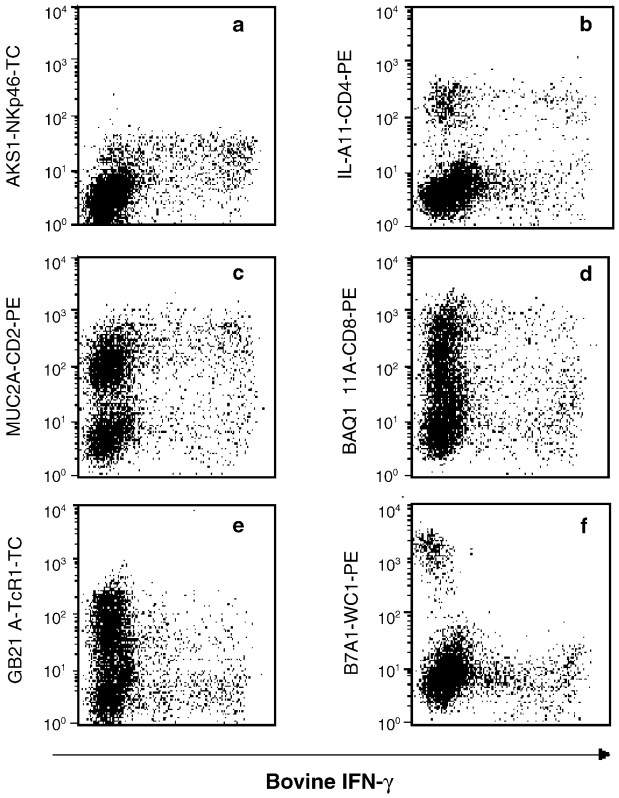

Figure 2 Phenotype of spleen cells producing IFN-γ after overnight stimulation with 20 ng/mL rHuIL-12 plus 1000 pg/mL rHuIL-18. Spleen cells were labelled with phenotype-specific monoclonal antibodies and then internally labelled with FITC-anti-bovine IFN-γ. (a) NKp46$^+$ spleen cells; (b) CD4$^+$ spleen cells; (c) CD2$^+$ spleen cells; (d) CD8$^+$ spleen cells; (e) TCR1$^+$ (γδT-cell) spleen cells; (f) WC1$^+$ (subset of γδT-cell). Resulting plots are representative of all calves.

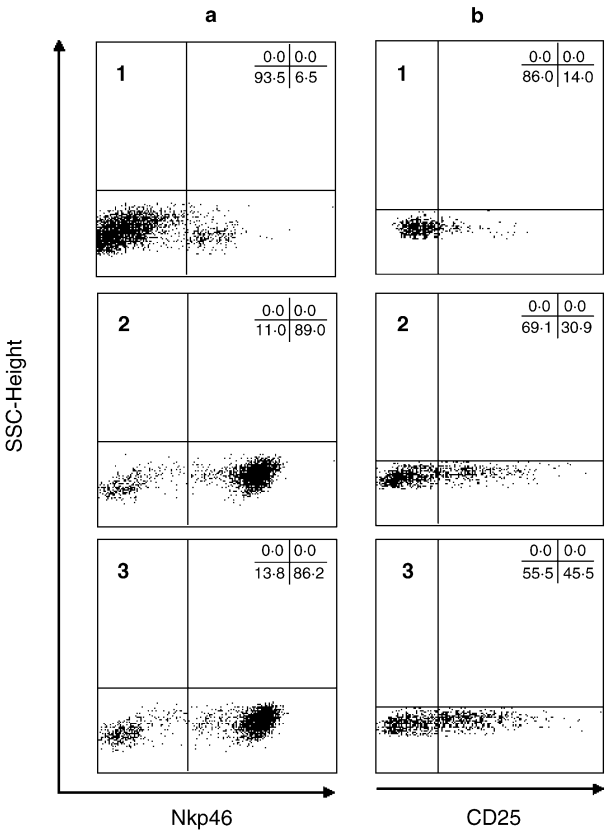

Figure 3 *In vitro* proliferation of bovine splenic NK cells (a) and expression of CD25 (b) after 14 days in culture in the presence of 2·5 U rBoIL-2 or 20 ng/mL rHuIL-15. Cells were labelled for flow cytometric analysis with AKS1 MoAb specific for NK cells (a) and CACT108A MoAb specific for CD25 (b). Fresh *ex vivo* spleen cells (a, panel 1, and b, panel 1) were compared with cells incubated for 14 days with rBoIL-2 (a, panel 2 and b, panel 2) or rHuIL-15 (a, panel 3 and b, panel 3). Numbers associated with the two lower quadrants represent the change in proportion of labelled cells after 14 days. Resulting plots are representative of all calves.

Culture-enriched splenic NK cells respond to IL-12 and IL-18 and produce IFN-γ

For some experiments, AKS1 MoAb was used to positively select splenic NKp46$^+$ NK cells from total spleen cell preparations or from IL-15-activated, 14 day cultures. However, NKp46 purified or 2 week old, IL-15-activated, highly enriched NK cell cultures without further purification yielded similar results, and thus enriched cultures were used for the majority of experiments. A small proportion of NK cells produced IFN-γ in response to IL-12 or IL-18 alone (Figure 5). However, the synergistic effect of IL-12 in combination with IL-18 was again pronounced with the majority of the NK cells producing IFN-γ (Figure 5). Stimulation with IL-18, with or without IL-12, resulted in an increase in the number of CD25$^+$ NK cells (Table 1).

Exposure of monocytes to *B. bovis* merozoites provides activation for NK cells and subsequent IFN-γ production via IL-12

Previous studies revealed that IL-12 mRNA expression in the spleen was up-regulated early in the response of calves to *B. bovis* infection. This was followed immediately by an increase in both IFN-γ mRNA and protein expression that ultimately resulted in a babesiacidal nitric oxide burst (1). To demonstrate that IL-12 from *B. bovis*-activated monocytes induce the innate production of IFN-γ, supernatants from merozoite-exposed monocytes were first evaluated for the presence of IL-12 using a dot-blot assay and then added to culture-enriched NK cells in the presence of a sub-optimal amount of exogenous IL-18. Figure 6 shows that

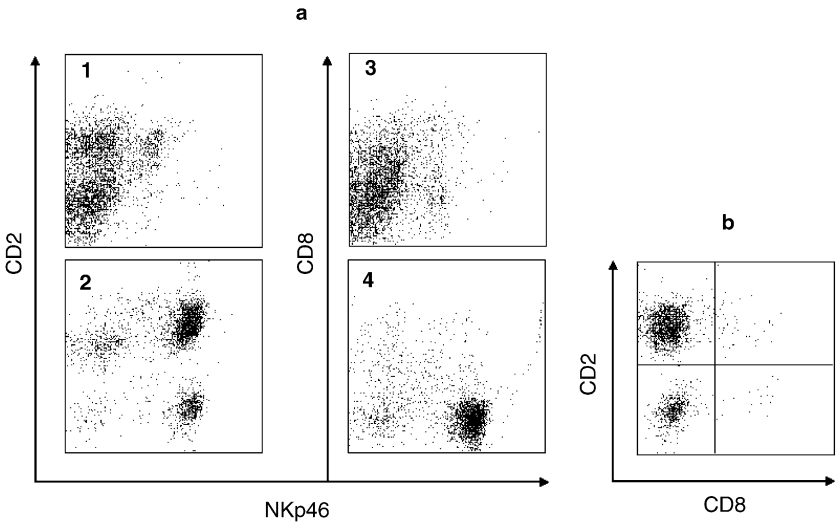

Figure 4 Three-colour flow cytometric phenotype analysis of bovine splenic NK cells obtained after 14 days in culture with 20 ng/mL rHuIL-15. (a) Spleen cells were labelled with AKS1 MoAb specific for NKp46, MUC2A MoAb specific for CD2, and BAQ111A MoAb specific for the alpha subunit of CD8. CD2 expression associated with NK cells from fresh (a, panel 1) and IL-15-activated (a, panel 2) spleen cells and similarly, CD8 expression associated with fresh (a, panel 3) and IL-15 activated (a, panel 4) NK cells. (b) CD2 vs. CD8 expression shown by gating on IL-15-activated NKp46$^+$ NK cells. Resulting plots are representative of all calves.

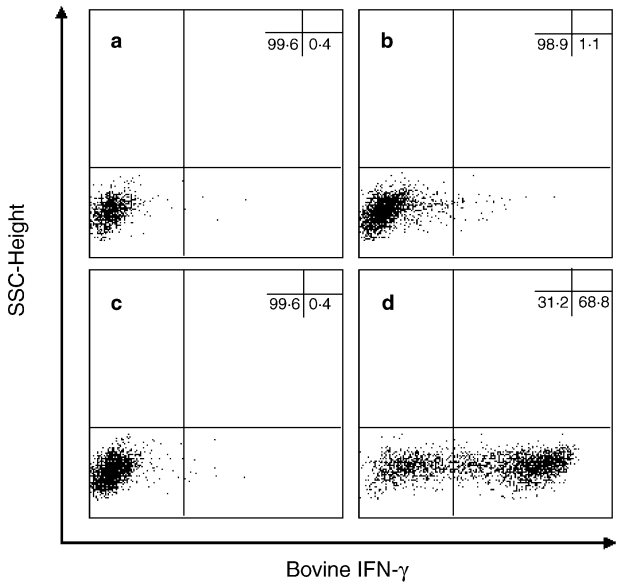

Figure 5 IFN-γ production in bovine spleen cells after IL-15 activation and IL-12 and/or IL-18 stimulation. Total spleen cells were cultured in 20 ng/mL rHuIL-15 for 14 days. The actively proliferating population at 14 days was 90% NK cells as described in Figure 3. The 14-day-old cultures were stimulated overnight with medium (unstimulated) (a), 20 ng/mL rHuIL-12 alone (b), 1000 pg/mL rHuIL-18 alone (c), or IL-12 plus IL-18 (d). Cells were internally labelled with FITC-anti-IFN-γ. Resulting plots are representative of all calves.

Table 1 Proportions (in percentages) of NKp46$^+$ cells expressing CD25 following stimulation with IL-12 and/or IL-18

	Medium	IL-12	IL-18	IL-12 + IL-18
Medium	13·4 (4·1)	23·3 (4·1)	48·8 (13·5)	55·1 (13·8)
IL-2	24·3 (6·4)	24·4 (6·9)	48·5 (15·5)	50·5 (10·0)
IL15	31·3 (11·5)	26·7 (16·6)	42·0 (15·7)	56·3 (17·2)

Values represent the proportion of IL-2- or IL-15-activated NK cells expressing CD25 after stimulation with IL-12, IL-18 or both from four animals.

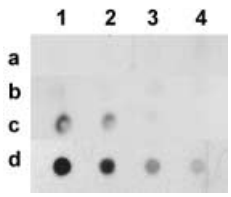

Figure 6 Parasite induction of IL-12 from monocytes. Dilutions of supernatants collected from monocyte cultures exposed to *Babesia bovis* merozoites were evaluated for IL-12 using a dot blot procedure with reactions normalized against a human IL-12 standard. Dilutions (columns 1–4) of monocyte supernatants from: (a) media control, (b) monocyte supernatant control, and (c) *B. bovis*-exposed monocyte supernatant, were compared to an IL-12 standard (d) to determine approximate levels of IL-12 p40. d1, 20 ng/mL, d2, 10 ng/mL, d3, 5 ng/mL and d4, 2·5 ng/mL.

B. bovis-exposed monocyte supernatants contained approximately 7 ng/mL of IL-12. This amount was sufficient to drive the production of IFN-γ from NK cells, as determined directly by an IFN-γ-specific ELISA (89·6 U/mL), and by internal labelling and flow cytometry (Figure 7g–i). 5·3% of the NK cells produced IFNγ in response to *B. bovis*-exposed monocyte supernatant (Figure 7h) compared to 0·5% exposed to control monocyte supernatant (Figure 7e). CC301 MoAb failed to neutralize IL-12 activity from these supernatants regardless of the amount used (data not shown). Therefore,

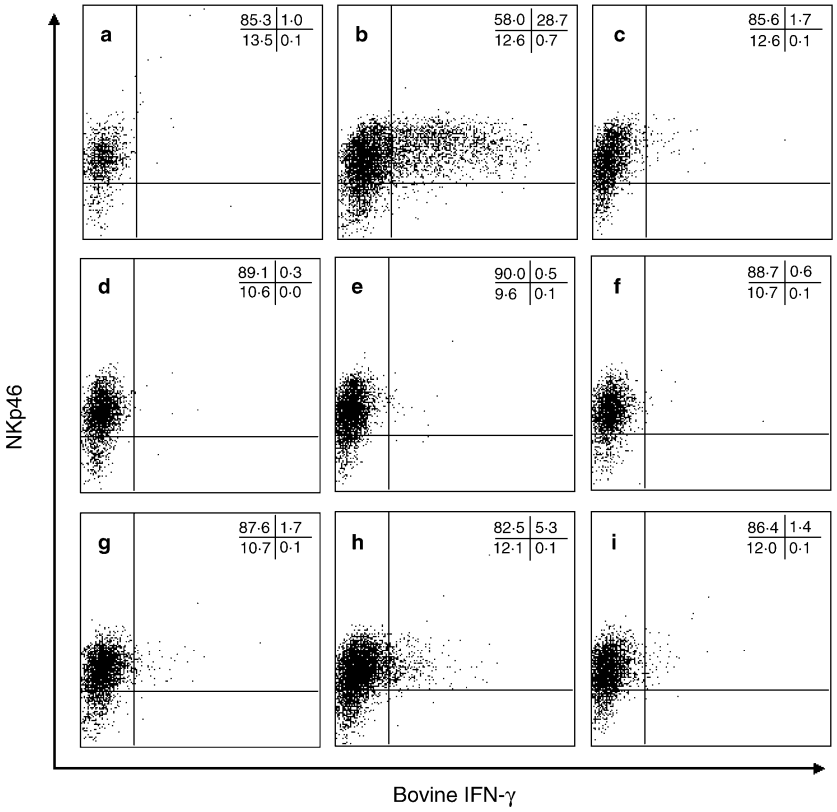

Figure 7 Supernatants from monocytes cultured with *Babesia bovis* contain IL-12 sufficient to stimulate IFN-γ production in bovine splenic NK cells. Fourteen day, rHuIL-15-enriched and activated NK cells were stimulated with: medium control (a); 20 ng/mL rHuIL-12 + suboptimal concentration of 200 pg/mL rHuIL-18 (positive control) (b); 20 ng/mL rHuIL-12 + suboptimal concentration of rHuIL-18 + 10 μM SB203580 inhibitor (c); control unexposed monocyte supernatant (d); control unexposed monocyte supernatant + suboptimal concentration of rHuIL-18 (e); control unexposed monocyte supernatant + suboptimal concentration of rHuIL-18 + 10 μM SB203580 inhibitor (f); *B. bovis* exposed monocyte supernatant (g); *B. bovis* exposed monocyte supernatant + suboptimal concentration of rHuIL-18 (h); and *B. bovis* exposed monocyte supernatant + suboptimal concentration of rHuIL-18 + SB203580 inhibitor (i). Cells from culture were stained with AKS1 (*Y*-axis) and labelled internally with FITC-anti-bovine IFN-γ (*X*-axis). Numbers in quadrants shown in upper right of each panel represent the proportions of total cells found in each actual defined quadrant for each panel. Plots are representative of three experiments.

SB203580, an inhibitor of MAPK-p38, an essential kinase in the IL-12/IL-18-dependent IFN-γ induction pathway in NK cells was used successfully to inhibit IFN-γ production (Figure 7c,f,i).

DISCUSSION

The importance of IFN-γ in the protective immune response of cattle against *B. bovis* infection is well established and involves IFN-γ-dependent, mononuclear phagocyte-mediated type-1 mechanisms. These include the induction of a transient, but effective nitric oxide response and the direction of the production of opsonic IgG_2 antibody (26). The source and conditions affecting IFN-γ production in the spleen, where haemoparasitic infections like babesiosis are resolved, has not been fully elucidated. A number of different cells, including αβ and γδ T-cells and NK cells, have been shown to produce IFN-γ under various conditions. However, during the innate response in calves to a virulent *B. bovis* infection, NK-like cells ($CD3^-$, $CD2^+$, $CD8^+$), unlike $CD3^+$ αβ and γδ T-cells, proliferated in the spleen (21). Although experiments were not designed to reveal the source of IFN-γ in that study, the proliferation of NK-like cells was coincident with the increase in plasma levels of IFN-γ and followed the increase in splenic mRNA levels for both IFN-γ and IL-12 (1).

In the present study, several different bovine splenic leucocyte subsets were shown to produce IFN-γ in response to IL-12 and IL-18, including an $NKp46^+$ NK cell. NKp46 expression was low in freshly obtained splenic NK cells, but up-regulated upon culture in IL-2 or IL-15, confirming similar responses from human NK cells (27) and bovine blood-derived NK cells (20). Not only did splenic NK cells proliferate under these conditions, resulting in a 15-fold increase in 2 weeks, but the staining intensity of the population was increased, indicating greater NKp46 expression on a per cell basis.

The IL-2 receptor on NK cells consists of an inducible α subunit (CD25), and constitutive β (CD122), and γ (CD132) subunits. The expansion of NK cells in culture appears to be independent of the α subunit, since IL-2 or IL-15 activation of NK cells resulted in a range of CD25 expression, with a substantial population of $CD25^-$ NK cells among the enriched population. IL-18 increased the proportion of NK cells expressing CD25 well above that of IL-2 or IL-15 activation, indicating that IL-18 may enhance receptor complex formation. In addition, the majority of IFN-γ production in response to IL-12 and/or IL-18 stimulation involved NK cells expressing CD25.

Fresh bovine splenic NK cells phenotypically resembled those obtained from peripheral blood. However, $CD2^+$, $CD8^-$ cells increased after IL-2 or IL-15 activation, in contrast to

blood-derived NK cells, where $CD8^+$ cells increased (20). CD8 expression on blood-derived NK cells varies depending on species, and based on our observations, may also vary depending on the tissue source of NK cells. Alternatively, CD8 expression in this case may simply be an *in vitro*-associated phenomenon. We previously described a $CD8^+$ NK-like cell proliferating in the spleen from calves responding to a *B. bovis* infection. The splenic milieu during infection may include modulatory elements not present in *in vitro* cultures that could influence CD8 expression.

It was clear from this study that IL-12 produced from *B. bovis* merozoite-exposed monocytes was functional, and served as the synergistic co-stimulant with IL-18 for the innate production of IFN-γ from NK cells. A similar circumstance has been reported with *Plasmodium*-stimulated PBMC cultures, where identifiable NK cells responded with the production of IFN-γ in a cytokine-dependent manner requiring both IL-12 and IL-18 (28). It has also been recently shown that rBoIL-12 was only partly responsible for the induction of IFN-γ production from bovine NK cells in response to mycobacterial antigens, suggesting that a co-stimulant might be involved (29). Attempts to neutralize IL-12 activity in *B. bovis*-exposed monocyte supernatants with CC301 MoAb failed. This MoAb was previously shown to completely neutralize recombinant bovine IL-12, but was unable to neutralize the majority of IFN-γ-inducing activity of endogenously produced bovine IL-12 (29), which probably explains the inability of CC301 MoAb to neutralize IL-12 activity in our experiments. Therefore, since IFN-γ production from NK cells induced by IL-12/IL-18 requires the MAPK p38 signalling pathway, SB203580, a specific inhibitor of p38 (30) was used to demonstrate the cytokine-specific induction of IFN-γ production from NK cells stimulated with the *B. bovis*-exposed monocyte supernatant.

The importance of IFN-γ to the resolution of an initial *B. bovis* infection has been well established (1,3,8) and is considered to be of importance in the spleen, since susceptibility to clinical disease is increased in splenectomized animals. We have previously shown that *B. bovis* merozoites plus exogenous IFN-γ stimulated purified monocyte production of nitric oxide (3,5,8) and that IFN-γ from total spleen cell preparations exposed to *B. bovis* merozoites stimulated the production of nitric oxide (1). This information, together with our recent data, suggests that splenic NK-cells may play an important role in the response to infection, serving as a major source of IFN-γ. This was most apparent in young calves, where induction of IL-12 and IFN-γ preceded the induction of IL-10, as compared to more susceptible adult cattle, where both IL-12 and IL-10 induction occurred simultaneously (1). In the study reported here, the amount of parasite-induced, endogenously produced bovine IL-12 was 30% less than the exogenously added rHuIL-12, yet the proportion of NK cells producing IFN-γ was only 17% of the proportion that responded to the exogenously added rHuIL-12. Although there may not be a linear relationship, it may also be that the monocyte supernatant contained some IL-10, sufficient to reduce the effects of 7 ng/mL of bovine IL-12 but not 20 ng/mL of rHuIL-12, despite the species-homologous source of the endogenous IL-12. We have previously demonstrated that monocytes do produce IL-10 when placed in culture and down-regulate the production of IFN-γ (31). Regardless, these studies identified a potentially important regulatory role for splenic NK cells in the innate immune response to *B. bovis*. There is also increasing evidence for their regulatory role in shaping the adaptive response, where NK cells have been shown to enhance expansion and function of $CD8^+$ lymphocytes (32). Therefore, our continuing studies will be designed to further clarify the interaction of splenic NK cells with other cells, such as dendritic cells and lymphocytes, in respect to infection with *B. bovis.*

ACKNOWLEDGEMENTS

We thank Dr Nancy Magnuson for the generous gift of rBoIL-2, Dr Chris Howard for providing the IL-A11 MoAb and Dr Jayne Hope for CC301 MoAb. We also thank Kim Kegerreis and Paul Lacy for excellent technical support, and Ralph Horn and Duane Chandler for animal care and sample acquisition. This study was supported by USDA-ARS-ADRU-CRIS no. 5348-32000-010.

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